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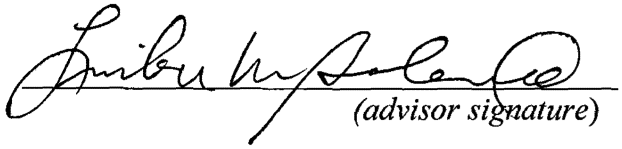

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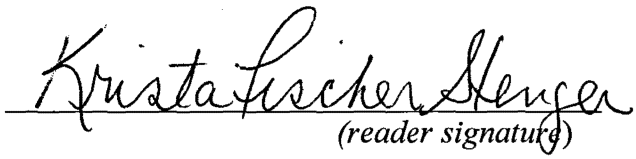

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Modulation of Fast-Inactivating Potassium Channels by Accessory Subunits and Fatty Acids: A Role for Calcium?

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Potassium channel interacting proteins (KChIPs) co-assemble with Kv4 α subunits to form native complexes that encode considerable components of neuronal somatodendritic A-type K^+ current (I_{SA}) in neurons, and transient outward current (I_{TO}) in cardiac myocytes. The binding of KChIPs to the cytoplasmic N-termini of Kv4 α subunits enhances surface expression by facilitating intracellular trafficking, stimulates subunit assembly, and regulates the functional gating properties of Kv4 channels. KChIPs, like many other neuronal calcium sensor proteins, contain four EF-hand calcium binding sites; two of which (EF-3 and 4) have particularly high affinity for calcium. Because Ca^{2+} is an important signaling molecule for cell physiology, it is suggested that Ca^{2+} may be an important factor for KChIP interactions with Kv4. In addition, it has been observed in many studies that polyunsaturated fatty acids (PUFAs), such as docosahexanoic acid (DHA) and arachidonic acid (AA) modulate A-type peak current and inactivation kinetics. In this study, we tested the hypothesis that PUFA modulation of Kv4/KChIP peak current and kinetics is sensitive to internal Ca^{2+} concentration. Using 50 μM BAPTA-AM to lower intracellular Ca^{2+}, results showed no difference of Kv4/KChIP kinetics or modulation by DHA among cells recorded under normal Ca^{2+} and low Ca^{2+}. These results were confirmed in cells expressing mutant forms of KChIP2c, which lacked either high affinity Ca^{2+} binding site EF-3 or 4. Control cells injected with mSlo, a channel activated by intracellular Ca^{2+}, exhibited a rightward shift of activation when treated with BAPTA-AM, suggesting that this treatment was sufficient. To test the effects of high intracellular Ca^{2+}, cells were incubated in 10 μM A23187, a calcium ionophore. Under increasing Ca^{2+} conditions (0.1, 0.5, 1.0, and 1.8 mM), there was no apparent change in Kv4/KChIP activation kinetics. mSlo- injected cells displayed a leftward shift of activation when treated with A23187 and were used as a positive control. Together, our results suggest that Kv4/KChIP kinetic properties and PUFA modulation are not directly sensitive to intracellular Ca^{2+} levels.

Voltage-gated potassium channels of the Kv4 family are composed of tetramers of α subunits (Yu et. al., 2005) and potassium channel interacting protein (KChIP) β auxiliary subunits (An et al., 2000), and are expressed in brain and heart (An et al, 2000). Kv4 currents activate upon membrane depolarization and then rapidly undergo voltage-dependent inactivation. Sustained voltage changes and/or repetitive activation promote accumulation of inactivation, which modulates activity-dependent changes in cellular excitability (Dodson and Forsythe, 2004). Changes in the rate and mechanism of inactivation, caused by modification of gating by KChIPs, impact how these channels modulate membrane excitability, which could have implications for their roles in learning and memory (Ramakers and Storm, 2002), regulation of the heartbeat (Kuo et al, 2001), and pain sensitivity (Birnbaum et. al., 2004; Hu et. al., 2006). In addition to changes of the channel's kinetic and structural characteristics, other molecules in the cellular environment, such as polyunsaturated fatty acids (PUFAs), can interact with Kv4 channels to modulate its activity.

Polyunsaturated fatty acids (PUFAs), specifically docosahexanoic acid (DHA) and arachidonic acid (AA), have been found to alter the excitability of surrounding cells, commonly by

disturbing the conformational changes of ion channels (Boland and Drzewiecki, 2008). AA is a lipid-soluble unsaturated fatty acid, and is also an important second messenger in a wide variety of cell types (Keros and McBain, 1997). Arachidonic acid is released from membrane phospolipids in concentrations higher than normal under pathological conditions, such as ischemia, epilepsy, or stroke (Villarroel and Shwarz, 1996). The activation of certain G-protein coupled metabotropic receptors and three different phospholipase enzymes (PLA_2, PLC, PLD) are capable of initiating the release of PUFAs from the membrane. Once released, PUFAs can be quickly metabolized to produce potent intermediates, such as eicosanoids, or can directly act as retrograde or anterograde messengers (Boland and Drzewiecki, 2008).

PUFAs are important endogenous molecules to study with Kv4 because they have been shown to modulate native A-type K^+ currents (Keros and McBain, 1997; Colbert and Pan, 1999) and recombinant Kv4/KChIP currents (Holmqvist et. al., 2001). Analysis of voltage clamped currents has shown that PUFAs significantly and reversibly inhibit K^+ peak current, the maximum amplitude of current passing through the channel. It is suggested that Kv4 current inhibition is caused directly by PUFAs themselves because the inhibitory effect is generally mimicked by the non-metabolizable eicosatetraynoic acid (ETYA) (Kehl, 2001; Drzewiecki, unpublished observation), not blocked by inhibitors of oxidative metabolism (Villarroel and Shwarz, 1996), and not mimicked by saturated and monounsaturated fatty acids (Honoré et al, 1994). Evidence suggests that PUFAs cause dual modulatory effects because in addition to inhibiting Kv4 currents, PUFAs also accelerate the time course of inactivation (Boland, Drzewiecki, Timoney, and Casey, submitted). However, it is unknown whether these effects on peak current and inactivation kinetics occur by similar or different mechanisms.

Although the dual modulatory effects of PUFAs on Kv4/KChIP have been reported, the mechanism(s) of their actions remains unclear and uninvestigated. Holmqvist et al (2001) hypothesized that KChIPs are responsible for the difference between AA current inhibition of native and heterologously expressed Kv4 channels, and that the kinetic modulation of Kv4 by AA is dependent on co-expression with KChIP. The results of the study showed that peak inhibition of Kv4.2 occurred regardless of the presence of KChIP subunits, whereas only Kv4.2 channels co-expressed with KChIP were kinetically modified by AA, suggesting that KChIP is required for PUFAs to accelerate the time course of inactivation. KChIP co-expression could be required for AA modulation of Kv4 channel inactivation gating if KChIPs are the molecular targets of AA. An alternative hypothesis, however, suggests that there exists a state-dependence to the modification of Kv4 by PUFAs, and that KChIP is not required to act as the molecular target of AA (Boland, Drzewiecki, Timoney, and Casey, submitted). Because structure and function are intimately related, it is crucial that KChIP structure be explored in order to gain a stronger understanding of its functional role.

KChIPs are cytoplasmic Kv4 channel interacting proteins that belong to the neuronal calcium sensor family of Ca^{2+} binding EF-hand proteins. These proteins co-assemble with Kv4 α subunits to form native complexes that encode neuronal somatodendritic A-type K^+ current (I_{SA}) in neurons, and transient outward current (I_{TO}) in cardiac myocytes (An et al, 2000; Kuo et al, 2001; Nadal et al, 2001). The binding of KChIPs to the cytoplasmic N-termini of Kv4 α subunits enhances surface expression by facilitating intracellular trafficking, stimulates subunit assembly, and regulates the functional gating properties of Kv4 channels (An et al, 2000; Holmqvist et al, 2002). Although these are the general properties of KChIP binding, some variation exists as there are four major KChIP isoforms (KChIPs1-4) encoded by multiple genes with several splice variants (An et al, 2000; Morohashi, 2002). Strong evidence from immunohistochemistry and co-immunoprecipitation analyses confirm the notion that KChIPs interact with Kv4 α subunits in brain membranes, revealing that KChIPs are critical constituents of native A-type complexes (Rhodes et al, 2004).

To help understand the nature of the interaction between Kv4 channels and KChIPs, studies have been performed to identify the possible KChIP binding sites on Kv4 channels. Using mutagenesis and X-ray crystallography, it has been shown that several regions in the Kv4.2 cytoplasmic amino terminus, residues 7-11 (Scannevin et. al 2004), 2-40 (Bähring et al, 2001) and 71-90 (Scannevin et. al 2004), are necessary for KChIP modulation and interaction with Kv4.2. Although the regions of KChIP binding have been identified on Kv4, it is not yet clear which specific structures of the KChIP molecule bind to the Kv4 channel. However, the recent co-crystal structure of Kv4.3 N-terminus and KChIP1 complex reveals two contact interfaces involved in the interaction (Pioletti et al, 2006; Wang et al, 2007). In the first interface, residues 6-21 of the N-terminal hydrophobic peptide of Kv4.3 reaches into a deep, elongated groove, and is sequestered by binding to the deep, hydrophobic pocket on the surface of KChIP1 (Wang et al, 2007).

This action causes the physical slowing of Kv4 inactivation by KChIPs. The association of KChIP1 with the Kv4.3 N-terminus at the second interface facilitates the stabilization of Kv4.3 tetrameric assembly (Wang et al, 2007). Residues E70 and F73 of the Kv4.3 N-terminus in the second interface are responsible for channel trafficking (Cui et al, 2008). Although we have a better understanding of the interaction of KChIPs with Kv4 N-termini, it is also suggested that other KChIP conserved structures, such as the amino terminus, conserved core region, and/or Ca^{2+} binding sites may be involved.

Evidence suggests that the kinetic effect of KChIPs on Kv4 inactivation may depend on the nature of the KChIP subunit and/or the internal Ca^{2+} concentration of the cell. Within the C-terminal domain of KChIPs (ex. KChIP 1, 2, 3), there exists a 70% conserved amino acid identity containing four EF-hand-like motifs. The term EF-hand was originally designated from the helix E-loop-helix F motif in the structure of the Ca^{2+} -binding protein parvalbumin (Branden and Tooze, 1998). All Ca^{2+} sensor proteins contain four EF hands (Braunewell and Gundelfinger, 1999). KChIPs, as well as others of the neuronal calcium protein family, contain the amino acids CPXG in the first EF-hand, which disables Ca^{2+} binding (Chen et al, 2006). Ca^{2+} binds with EF-hands 2, 3, and 4 (Osawa et al, 2005); however, EF-3 and 4 contain particularly high affinity binding sites (Chang et al, 2003; Osawa et al, 2005) at regions rich with the negatively charged amino acids glutamic acid and aspartic acid (Gifford et al, 2007). On the other hand, EF-2 does not bind Ca^{2+} with high affinity because it contains aspartic acid at the key 12-position glutamic acid of the EF-hand binding loop, which diminishes its binding selectivity of Ca^{2+} (da Silva et al, 1995). The EF-3 hand consists of an α1-helix, a β1-strand, and an α2-helix, whereas the EF-4 hand consists of an α3-helix, a β2-strand, and an α4-helix (Yu et al, 2007). Ca^{2+} ions bind at Ca^{2+} -binding loops and β-strand regions where highly negatively charged protein surfaces are located (Yu et al, 2007).

Because KChIP molecules contain Ca^{2+}-binding EF hands, it is rationally hypothesized that Kv4/KChIP function may be regulated by intracellular Ca^{2+} signaling. Typical concentrations of intracellular Ca^{2+} range from approximately 80-120nM in resting cells (Nakada and Mizuno, 1998), and can be increased by Ca^{2+} release from intracellular stores or influx through a variety of plasma membrane ion channels (Taylor et al, 2008). The driving force for calcium entry is the result of an electrochemical gradient between the extracellular (1.3-2 mM) and intracellular concentrations (Taylor et al, 2008). Interestingly, KChIP 2.2 binds to the Kv4 N-terminus regardless of the presence or absence of Ca^{2+} (Chen et al 2006), but an increase in Ca^{2+} concentration strengthens KChIP interactions with Kv4 channels (Chen et al 2006). It has also been demonstrated that the removal of EF-4 using a truncated KChIP2.2 abolished KChIP2.2 binding to Kv4 in the absence of Ca^{2+}, but the subunit interaction could be recovered with the addition of Ca^{2+} (Chen et al 2006). These observations suggest that EF-4 is essential to KChIP binding interactions with Kv4, and that when Ca^{2+} concentrations are increased, Ca^{2+} is bound by EF-3, the other high affinity site on KChIP. This implies that EF-3 Ca^{2+} -binding rescues KChIP/Kv4 interactions in the absence of EF-4. EF-2 would most likely not be responsible for the rescue because it has much less affinity for Ca^{2+} than EF-3. In addition, it is also suggested that KChIP-mediated modulation of Kv4.2 kinetics is dependent on Ca^{2+} or is highly sensitive to point mutations within EF-hands (An et al, 2000). Patel and colleagues (2004) have shown that the slow time constant of inactivation of Kv4/KChIP can be altered with the application of a membrane-permeant Ca^{2+} chelator. Together, the evidence implies that there exists a critical link between KChIPs in the cytoplasm, their EF-hands, and intracellular Ca^{2+} concentrations which renders KChIPs capable of binding to Kv4, and subsequently altering Kv4 kinetic properties.

Based on evidence that PUFAs and intracellular Ca^{2+} are both important factors for Kv4/KChIP channel modulation, studies were performed to test the hypothesis that PUFA modulation of Kv4/KChIP peak current and kinetics is sensitive to internal Ca^{2+} concentration. It was predicted that exposure to a low Ca^{2+} environment would decrease PUFA modulation of Kv4/KChIP channels because a decrease in intracellular Ca^{2+} would weaken KChIP interactions with Kv4. If KChIP does not bind well to Kv4, the inactivation kinetics of Kv4 would remain fast. Therefore, PUFA would be unable to accelerate Kv4 inactivation any further, and peak inhibition would be weakened. Following the same reasoning, it was predicted that in a high Ca^{2+} environment, KChIP interactions with Kv4 would be much stronger, inducing slower Kv4 inactivation, and thus, greater PUFA modulation could occur. In order to decrease intracellular Ca^{2+} concentrations, prior to recordings, cells were incubated in BAPTA-AM, a membrane-permeable, selective chelator of intracellular Ca^{2+} stores (Ng et al, 1988). We predicted this would act as a sponge to bind any free Ca^{2+} entering the cytoplasm. In addition, to further understand the impact of low Ca^{2+} concentration on Kv4/KChIP interactions and PUFA modulation, studies were performed in cells that were

co-injected with Kv4 and KChIPs containing mutants for either EF-3 or EF-4, in order to minimize Ca^{2+} binding to KChIPs. To raise intracellular Ca^{2+} concentrations, prior to recordings, cells were incubated in A23187, an ionophore which increases the Ca^{2+} permeability of biological membranes (Reed and Lardy, 1972). Along with A23187 treatment, we also manipulated the extracellular Ca^{2+} levels of solutions, which would alter how much Ca^{2+} crossed the membrane and entered the intracellular environment. Together, these experiments tested the impact of Ca^{2+} concentrations on Kv4/KChIP kinetic properties and PUFA modulation. These experiments are important because they contribute knowledge to the complex study of the modulation of native K^+ channels by substances in their natural environment, such as PUFAs and Ca^{2+}, and provide us with insight to the potential roles of these interactions in learning, memory, and brain trauma.

MATERIALS AND METHODS

Preparation of RNA. Plasmids containing the cDNAs encoding for rat Kv4.2, rat KChIPs (isoforms 1b and 2c), and mSlo were linearized and purified with Geneclean, and capped RNAs were synthesized *in vitro* using Ambion(Applied Biosystems, Austin, TX) mMessage mMachine RNA polymerase kits. RNA was purified by use of the RNAid kit (Bio 101, Vista, CA) or RNeAsy (Qiagen, Valencia, CA) and stored at -80°C in DEPC-treated water. The size of transcription reaction products were verified by agarose gel electrophoresis, and RNA concentrations were determined by spectrophotometry using the equation: 1 A_{260} = 40 µg/ml RNA.

Expression of ionic currents in oocytes. Oocytes were harvested from *Xenopus laevis* (Xenopus I, Dexter, MI), previously injected with human chorionic gonadotropin. Female frogs were anesthetized by immersion in 0.1% 3-aminobenzoic acid ethyl ester (Sigma-Aldrich) buffered to pH 7 with sodium bicarbonate. Ovarian lobes were surgically removed from a small abdominal wound, which was sutured under anesthesia. Oocytes were released by gentle agitation for 20-60 minutes in 0.5 mg/ml collagenase 1A (Sigma-Aldrich) dissolved in a Ca^{2+}-free OR-2 solution containing (in mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES, pH 7.4 with NaOH. Oocytes were extensively washed with Ca^{2+}-free OR-2, and then with ND-96 (in mM): 96 NaCl, 1 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, and 2 Na pyruvate, pH 7.4 -7. 5 with NaOH with 50 U/ml penicillin G and 50 ug/ml streptomycin. Stage V and VI oocytes were collected using a dissecting microscope. De-folliculated oocytes were injected the same day or the following day with 50 nl of RNA dissolved in DEPC-treated water. Kv4 RNAs were injected alone at 10-40 ng, or at 5-16 ng per oocyte when mixed with KChIP RNAs at a concentration designed to yield a 1:4 molar ratio in order to saturate α with β subunits. Oocytes were maintained in ND-96 solution at 19°C. Electrophysiological recordings were performed 2-5 days post-injection.

Data acquisition and analysis. Potassium currents were recorded from oocytes by two-electrode voltage clamp using a Geneclamp 500B amplifier (Axon Instruments, Foster City, CA). Voltage-measuring and current-passing electrodes were filled with 3 M KCl and had resistances between 0.3 – 1.0 MOhm. Currents were sampled at 5-10 kHz and filtered at 1-2 kHz. All recordings were done at room temperature (22-23 °C). Oocytes were voltage-clamped at -90 mV and voltage protocols were applied to measure peak current and kinetic features of channel gating, as described in the Results and Figure Legends. Oocytes were perfused continuously with a low chloride external recording solution (to minimize endogenous oocyte Cl^- currents) containing (in mM): 96 Na methanesulfonate (MES), 2 K MES, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.5 with NaOH. Agar bridges were used to connect the bath to separate wells containing the grounding wires immersed in 3 M KCl. External solution changes were made by manually switching a valve that controlled gravity-driven solution flow across the oocyte recording chamber. Data were recorded on Pentium computers equipped with Digidata 1320A (Axon Instruments-Molecular Devices) A/D hardware. Axon's Clampex acquisition (v.8) and Clampfit analysis software (v.9) were used. In most protocols, leak subtraction with P/-4 or P/-6 pulses from a holding potential of –90 mV was used. Data were transferred to Microsoft Excel and Microcal Origin v. 5 (Northhampton, MA) for analysis, curve-fitting, and the production of Figures. Data are expressed as means ± SEM.

Reagents. Reagents were obtained from Sigma-Aldrich (St. Louis, MO). Docasahexaenoic acid (DHA) was dissolved in ethanol at 1000-3000X, stored at -20°C, diluted into recording solution immediately prior to use, and replenished every 2 hours or sooner. 1,2-bis-(o-Aminophenoxy)-ethane-N,N,-N',N'-tetraacetic acid tetraacetoxy-Methyl ester (BAPTA-AM) was dissolved in DMSO at 1000X, stored at -20°C, and diluted into ND-96 prior to use. A23187 was dissolved in DMSO at 1000X, stored at -20°C, and diluted into low Cl^- recording solution (at 0.1 mM Ca^{2+}, 2.7 mM Mg^{2+}) prior to use. When recording solutions were made with increasing Ca^{2+} concentrations, the total external divalent cation

concentration was kept constant at 2.8 mM in order to avoid membrane surface charge effects.

RESULTS

mSlo as an intracellular Ca^{2+} reporter. In order to develop the protocols for the use of BAPTA-AM and A23187 as agents to alter intracellular Ca^{2+} concentrations of oocytes, studies were first performed using cells injected with RNA encoding for the mSlo Ca^{2+}-activated potassium channel. We selected mSlo because it could serve as a reporter for increases in intracellular Ca^{2+} concentrations since increased intracellular Ca^{2+} activates these channels (McManus, 1991). BAPTA-AM was used in this study because the presence of the acetoxymethyl ester moiety renders the molecule membrane permeable, which enables it to serve as a sponge for released intracellular Ca^{2+}. As shown in Figure 1 A and C, it was determined that sixty minutes of exposure to 50 μM BAPTA-AM, whether by incubation (A) or direct bath application every ten minutes (C), was the appropriate amount of time needed to sufficiently lower internal Ca^{2+} concentration because this amount of time produced the maximal rightward shift observed for the activation of mSlo. Also, additional time of exposure did not further shift the G-V curve (Fig. 1C).

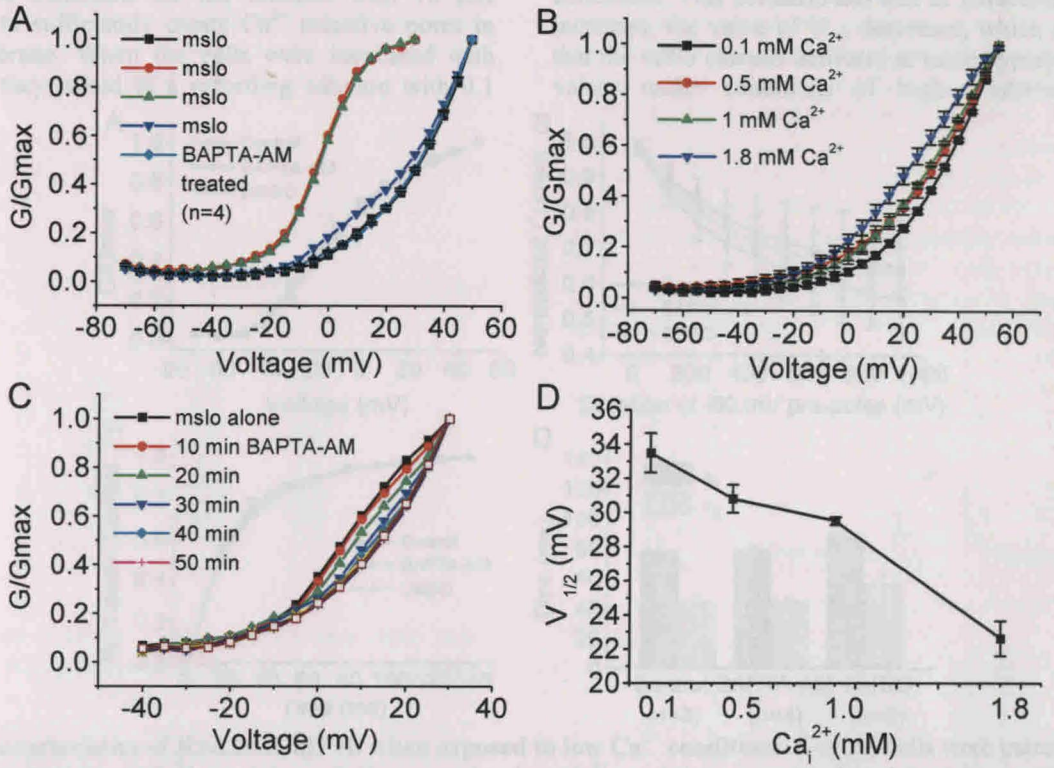

Fig.1 Test of the effects of intracellular Ca^{2+} concentration on mSlo activation. Oocytes were injected with RNA encoding for the mSlo Ca^{2+} activated potassium channel. (A) Graph showing activation data of 4 different untreated cells expressing mSlo, and average data of cell treated with BAPTA-AM (n=4). Intracellular Ca^{2+} concentration was reduced by incubating cells for 1 hour in 50 μM BAPTA-AM prior to recording. The data for the two mSlo control cells that were the most left-shifted were normalized to the conductance values at +30 mv, rather than +50 mV, because activation plateaued at +30 mV. All other conductance curves were normalized with the conductance value at +50 mV. BAPTA-AM treated cells displayed a rightward shift of the activation curve. (B) Graph displaying mSlo activation analysis in the form of a G-V curve. Cells (n= 5 or 6) were incubated in 10 μM A23187 for 10 minutes in 0.1 mM [Ca^{2+}] recording solution prior to recording. During recordings of mSlo activation, solutions with increasing concentrations of Ca^{2+} (0.5, 1.0, and 1.8 mM) were applied externally. (C) Activation data acquired from a cell injected with mSlo where 50 μM BAPTA-AM was applied in the recording chamber every 10 minutes during the cell recording. (D) Activation curves under each intracellular Ca^{2+} condition were fitted to an exponential growth function, and from that $V_{1/2}$ values were obtained. Graph displays the magnitude of the voltage required to elicit 50% of the maximum conductance value under the specified Ca^{2+} conditions (0.1, 0.5, 1.0, and 1.8 mM) (n= 5 or 6). Data shown are average ± SEM.

However, it seems that for most cells, the resting Ca^{2+} level is relatively low to start, so there was little opportunity to decrease the concentration even more. In contrast, a few cells out of all that were tested had high resting Ca^{2+} levels to begin with, so a dramatic decrease in internal Ca^{2+}, as evidenced by a drastic rightward shift of activation, could occur (Fig. 1A).

Cells injected with RNA encoding for mSlo were also utilized to determine the appropriate conditions for raising intracellular Ca^{2+} with the Ca^{2+}-ionophore, A23187. Compared to the concentration and time required for BAPTA-AM, A23187 was much more potent and fast-acting. As determined by the left-shifted mSlo activation curves in Fig. 1B, cells only had to be incubated for ten minutes with 10 μM A23187, to sufficiently create Ca^{2+} selective pores in the membrane. When the cells were incubated with A23187, they rested in a recording solution with 0.1 mM Ca^{2+} and 2.7 mM Mg^{2+}, the lowest Ca^{2+} concentration used. While performing the electrophysiological recordings, the first activation protocol was run with the same low Ca^{2+} recording solution, and every ten minutes, a different recording solution with increasing Ca^{2+} concentration (0.5, 1.0, 1.8 mM) was used for the recording. The Ca^{2+} and Mg^{2+} concentrations were always balanced in order to keep the concentration of divalent cations at 2.8mM in order to avoid any membrane surface charge effects. The activation data from Fig. 1B were used to generate the curve in Fig. 1 D, which shows the magnitude of the voltage required to elicit half of the maximum conductance values under increasing internal Ca^{2+} conditions. This demonstrates that as intracellular Ca^{2+} increases, the value of $V_{1/2}$ decreases, which suggests that the mSlo channel activates at more hyperpolarized values under conditions of higher internal Ca^{2+}.

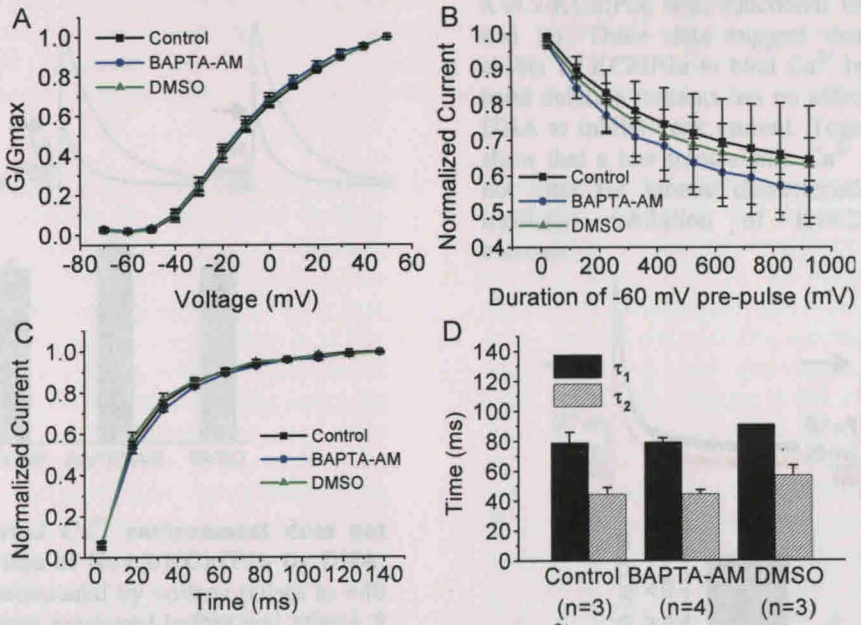

Fig. 2 Characteristics of Kv4.2/KChIP1b when exposed to low Ca^{2+} conditions. Control cells were untreated and recorded under normal Ca^{2+} conditions. Cells exposed to low Ca^{2+} conditions were incubated for 1 hour with 50 μM BAPTA-AM prior to recording. DMSO treated cells were incubated for 1 hour before recording. (A) G/Gmax curve showing normalized activation of Kv4/KChIP1b current stimulated by voltage pulses held at 0 mV for 20 ms, pulsing to -70 mV and increasing to +55 mV in 10 mV increments, each held for 500 ms. Peak currents from each voltage stimulation were measured, and converted to conductance (G) using the equation $G=(current/(V-E_k))$, with E_k= -85 mV. (B) Normalized data of current displaying the onset of closed state inactivation. Peak currents measured were at a resting voltage of -100 mV and then stimulated by a pulse to +40 mV for 500 ms, returned to -100 mV, stepped to a prepulse of -60 mV for 150 ms, and pulsed again to +40 mV for 125 ms. Ten pulses were given in this fashion with increasing duration of the pre-pulse. Peak currents were normalized against the maximum current for each recording. (C) Normalized data of current displaying the recovery from closed state inactivation. Cells were held at -90 mV for 140 ms, pulsed to +40mV for 1000 ms, -120 mV for 12 ms, and back to +40 mV for 200 ms. Peak currents were measured and then normalized against the maximum current. (D) Comparison of fast and slow inactivation time constants fitted to inactivation at +40 mV of cells treated with no treatment, BAPTA-AM, or DMSO. Current traces were fit to an exponential function with 2 time constants (τ_1 and τ_2) using the Chebyshev method. Data shown are averages ± SEM for Figures A-D (n=3 or 4).

Ca^{2+} does not modify Kv4.2/KChIP1b kinetics. The protocols for altering intracellular Ca^{2+} concentrations established by studies with mSlo-injected cells were employed for cells expressing Kv4.2/KChIP1b in order to determine if low Ca^{2+} causes changes in any basal kinetic properties of the channel. Any changes in these kinetic properties could represent Ca^{2+}-dependent modifications of KChIP interactions with Kv4.2. The data from Fig. 2 shows that there were no differences in activation (A), the onset of closed state inactivation (B), recovery from inactivation (C), and the fast and slow inactivation time constants (D) between cells that were untreated and those that were exposed to low intracellular Ca^{2+} conditions. Because there are no differences in the kinetic qualities, these data suggest that a low internal Ca^{2+} environment does not alter KChIP1b binding and modification of Kv4.2.

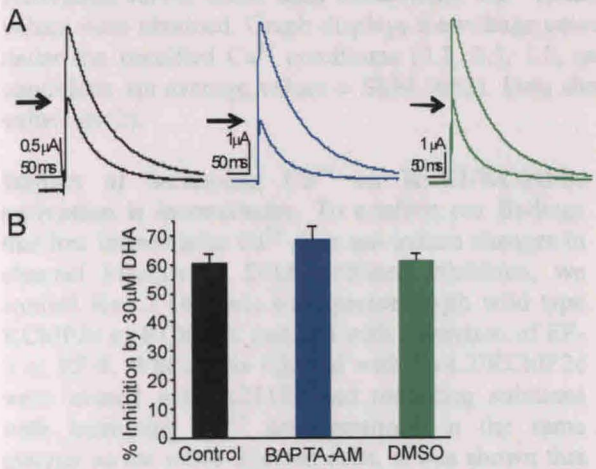

Fig. 3 Low internal Ca^{2+} environment does not alter the modulation of Kv4.2/KChIP1b by DHA. (A) Peak current stimulated by voltage pulses to +40 mV for 800 ms were measured before and after a 5 minute application of 30 µM DHA (depicted by arrow) and (B) represented in bar graph for control cells, and those incubated in 10 µM BAPTA-AM or DMSO. Data shown are averages ± SEM (n=4 for all conditions).

DHA modulation of Kv4.2/KChIP1b is not sensitive to low Ca^{2+}. In order to determine if low internal Ca^{2+} alters the modulation of Kv4.2/KChIP1b by DHA, a PUFA, peak currents measured at +40 mV and the amount of DHA-mediated peak current inhibition were compared for cells that were untreated and those incubated with 50 µM BAPTA-AM for sixty minutes prior to recording. Figure 3 (A and B) shows that the amount of DHA-mediated inhibition was approximately 60-70% for both control and BAPTA- AM treated cells. These data suggest that low internal Ca^{2+} does not alter the modulation of Kv4.2/KChIP1b by DHA.

KChIP2c EF-hand mutants are inhibited by DHA. Although the kinetics of EF-3 and EF-4 mutants were not studied plainly, the mutants were used in order to further study the effects of low intracellular Ca^{2+}. The co-expression of these mutated KChIPs generates conditions that mimic a low internal Ca^{2+} environment because when the EF-hand is mutated (EF-3: D171 and D175; EF-4: D219 and D223), there is no Ca^{2+} binding where that site would normally be, so KChIP interactions may be altered. When cells injected with Kv4.2 and co-expressed with KChIP2c EF-hand mutants were treated with 30 µM DHA, the peak current amplitude measured at +40 mV was inhibited by approximately 50%. This value did not differ from the amount of inhibition of Kv4.2/KChIP2c with functional EF-hands (Fig 4 A and B). These data suggest that minimizing the ability of KChIP2c to bind Ca^{2+} by introducing EF-hand deletion mutants has no effect on the ability of DHA to inhibit peak current. Together, these results show that a low intracellular Ca^{2+} environment does not alter the kinetic characteristics or the DHA-mediated inhibition of Kv4.2/KChIP channel currents.

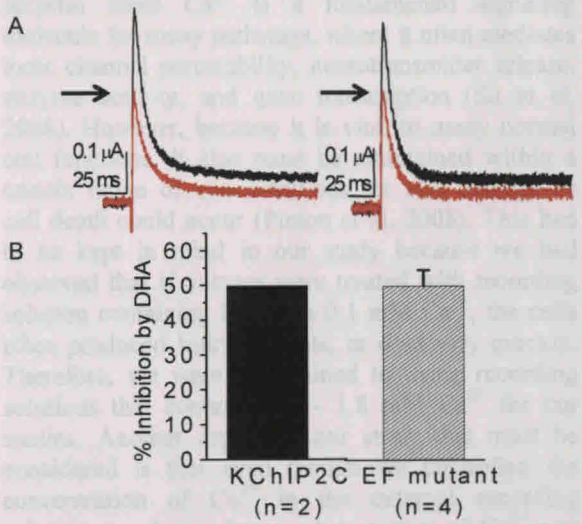

Fig. 4 The impact of mutation of EF hands of KChIP2C on PUFA inhibition. (A) Peak current stimulated by voltage pulses to +40 mV for 800 ms were measured before and after a 5 minute application of 30 µM DHA (depicted by arrow). (B) Bar graph displays the percent inhibition by 30uM DHA of currents pulsed to +40 mV for Kv4.2 co-expressed with wild type KChIP2C (n= 2) or KChIP2C with mutations of EF 3 or 4 (n=4). Data from EF3 and EF4 mutants were not different, so data were combined for the EF mutant bar. Data shown indicate average ± SEM.

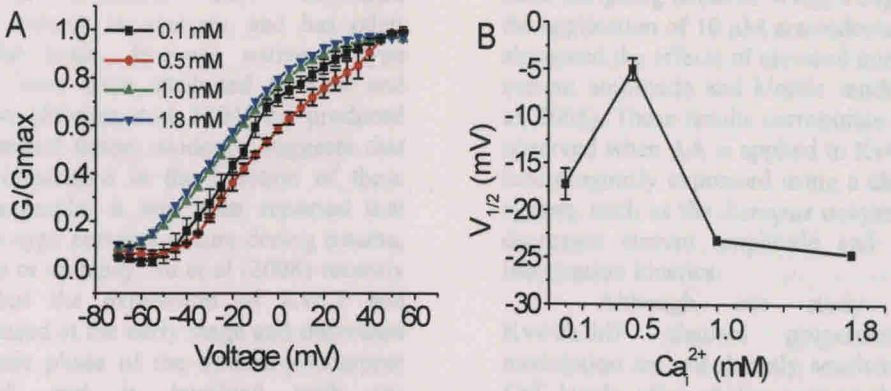

Fig. 5 Test of the effects of increasing intracellular Ca^{2+} concentration on Kv4/KChIP2c activation. (A) Graph displaying Kv4/KChIP2c activation analysis in the form of a G-V curve. Cells were incubated in 10 μM A23187 for 10 minutes in 0.1 mM [Ca^{2+}] low Cl^- recording solution prior to recording. During recordings of Kv4/KChIP2c activation, solutions with increasing concentrations of Ca^{2+} (0.5, 1.0, and 1.8 mM) were applied externally. (B) Activation curves under each intracellular Ca^{2+} condition were fitted to a sigmoidal function, and from that V1/2 values were obtained. Graph displays the voltage amount required to elicit 50% of the maximum conductance value under the specified Ca^{2+} conditions (0.1, 0.5, 1.0, and 1.8 mM). Data shown for cells in 0.1 and 0.5 mM Ca^{2+} conditions are average values ± SEM (n=3). Data shown for cells in 1.0 and 1.8 mM Ca^{2+} conditions are average values (n=2).

Impact of increasing Ca^{2+} on Kv4.2/KChIP2c activation is inconclusive. To confirm our findings that low intracellular Ca^{2+} does not induce changes in channel kinetics or DHA-mediated inhibition, we studied Kv4.2 channels co-expressed with wild type KChIP2c or KChIP2c mutated with a deletion of EF-3 or EF-4. When cells injected with Kv4.2/KChIP2c were treated with A23187 and recording solutions with increasing Ca^{2+} concentrations in the same manner as the mSlo-injected cells, it was shown that activation becomes slightly more left-shifted. After the application of solution containing 0.5 mM Ca^{2+}, the activation curve shifted a little to the right, and with applications of increasing Ca^{2+} concentration, the curve shifted to the left, past where it started at the lowest Ca^{2+} concentration (0.5 mM) (Fig. 5A). The data in Fig. 5 B show these results more clearly. From these data, it is difficult to draw a conclusion about the effects of increasing intracellular Ca^{2+} concentrations on Kv4.2/KChIP2C activation.

DISCUSSION

A fundamental conclusion of this study is that DHA modulation of heterologously expressed Kv4/KChIP kinetics and peak inhibition is not sensitive to intracellular Ca^{2+} concentration since the kinetic properties and mean magnitude of peak inhibition were not different among cells exposed to normal, low, and high Ca^{2+} concentrations. From these results, it can also be interpreted that the binding of KChIPs to Kv4 channels and the subsequent modulatory events due to their interactions are also not impacted by intracellular Ca^{2+}. These results and conclusions may come as a surprise since Ca^{2+} is a fundamental signaling molecule for many pathways, where it often mediates ionic channel permeability, neurotransmitter release, enzyme activity, and gene transcription (Su et al, 2008). However, because it is vital to many normal cell functions, it also must be maintained within a certain range of concentrations, or else damage or cell death could occur (Pinton et al, 2008). This had to be kept in mind in our study because we had observed that if oocytes were treated with recording solution containing less than 0.1 mM Ca^{2+}, the cells often produced leaky currents, or died very quickly. Therefore, we were constrained to using recording solutions that contained 0.1- 1.8 mM Ca^{2+} for our studies. Another aspect of our study that must be considered is that even though we controlled the concentration of Ca^{2+} in the external recording solution, we do not know what amount of Ca^{2+} was actually capable of generating an influx to the intracellular environment via Ca^{2+} channels. We implemented cells expressing mSlo as a positive control to minimize this limitation. However, others have alleviated this problem with the use of cellular Ca^{2+} indicators, such as aequorin (a phytoprotein), fluorescent dyes, and fluorescent proteins (Brownlee, 2000). In the future, our studies could be repeated and possibly made more precise with the use of such techniques.

As aforementioned, Ca^{2+} facilitates neurotransmitter release in neurons, and has other roles within the brain. Because native A-type currents, which have been attributed to Kv4 and KChIP complexes (Rhodes et al, 2004), are produced in excitable neuronal tissue, evidence suggests that Ca^{2+} may be implicated in the function of these channels. For example, it has been reported that modulation of A-type currents occurs during trauma, such as ischemia or epilepsy. Su et al (2008) recently demonstrated that the expression of Kv4.2 and KChIP1 is increased at the early stage and decreased during the chronic phase of the lithium-pilocarpine epilepsy model, and is involved with the development of epilepsy. In addition to changes in expression of Kv4.2 and KChIP1 following status epilepticus, when 10 μM 4-AP (a potassium channel blocker) was applied to CA1 hippocampal neurons, an abrupt 293% increase in intracellular Ca^{2+} occurred. These results suggest that 4-AP blocks Kv4.2, resulting in high intracellular Ca^{2+} and disrupted homeostasis. Such disruption is then suspected to alter Kv4.2 activity, which manifests as epileptic episodes. Interestingly, it is suggested that the increased expression of KChIP1 could be implicated so that it can serve as a Ca^{2+} buffer by binding Ca^{2+} with its EF hands in order to render the disruption of homeostasis less severe. Therefore, in this scenario, Kv4, KChIP, and Ca^{2+} each play a role that could potentially be exploited as treatment for epilepsy.

An additional study by Wang and colleagues (2005) also indicates a relationship between Kv4, KChIP, and Ca^{2+}, but this study also addresses a role for arachidonic acid. According to this study, an elevation of intracellular Ca^{2+} by the addition of 2 mM Ca^{2+} in the pipette solution caused an almost doubling of current amplitude and a slowing of inactivation; however, there was no effect on either the recovery from inactivation or the voltage-dependent steady-state activation properties of the A-current (Wang et al, 2005). The lack of impact of elevated intracellular Ca^{2+} on steady-state activation is similar to what we observed when additional Ca^{2+} was added to Kv4.2/KChIP2c because our results showed that the G-V curve was slightly right-shifted, and then left-shifted with the addition of more Ca^{2+}. This suggests that the altered shifting of the G-V curve is probably not different, and could average to generate a single curve. It is important to note that the effects of Ca^{2+} were not mimicked by Co^{2+} (Wang et al, 2005), which suggests that modulation is not simply due to a general divalent cation mechanism. This is a key observation because once again it suggests that KChIP and its Ca^{2+} -binding EF hands could be implicated in this mechanism. However, the most intriguing result of Wang's experiment was that the application of 10 μM arachidonic acid completely abrogated the effects of elevated intracellular Ca^{2+} on current amplitude and kinetic modulation (Wang et al, 2005). These results corroborate what is typically observed when AA is applied to Kv4/KChIP currents heterologously expressed using a channel expression system, such as the *Xenopus* oocyte model, where it decreases current amplitude and accelerates Kv4 inactivation kinetics.

Although our study indicates that Kv4/KChIP channel properties and PUFA modulation are not directly sensitive to intracellular Ca^{2+} levels, other studies suggest that the activity of Ca^{2+} is very complex, and may still be a key player for the interaction of KChIPs with Kv4 and PUFA modulation. In order to obtain a better understanding of these complex reactions, the approach we used in our study could be repeated with several modifications. For example, both EF-3 and EF-4 could be mutated in KChIP 2c in order to eradicate both high affinity Ca^{2+} binding sites. However, because KChIP is involved with the trafficking of Kv4 to the membrane, it is possible that the elimination of both high affinity sites could interfere with this function, as observed in triple mutants with EF-2, 3, and 4 mutations (An et al, 2000). Another idea would be to mutate EF-1 by altering the CPXG amino acid sequence, and/or EF-2 by mutating the aspartate to a glutamate at the 12th position of the EF-hand binding loop, so that they could become high affinity Ca^{2+} binding sites like EF-3 and 4. This could potentially double the Ca^{2+} binding ability of KChIP, which could increase its interactions with and modulation of Kv4. With the completion of such additional studies, our knowledge of neuronal excitability and function could be significantly enhanced, which could provide insight to the mechanisms of learning and memory, ischemia, and epilepsy.

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